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IMMUNOASSAY OF NEWCASTLE DISEASE VIRUS AND ANTI-NDV IN MOUSE SERUM USING A LIGHT-ADDRESSABLE POTENTIOMETRIC SENSOR (U)

by

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Immunoassay of Newcastle Disease Virus and Anti-NDV in Mouse Serum Using a Light-Addressable Potentiometric Sensor

by

Brenda Wikjord, H. Gail Thompson and William E. Lee

Suffield Memorandum 1423

ABSTRACT

A rapid nonradioactive sandwich immunoassay which utilizes biotin-streptavidin mediated filtration capture of immune complexes in conjunction with a silicon sensor was developed for the detection of virus and antibody to virus in serum. Using Newcastle disease virus (NDV) and mouse anti-NDV IgG spiked into mouse serum as a clinical-style model, the lower limits of detection (LOD) were determined. The LODs per test site were 1 ng for NDV and 4 ng for anti-NDV. The assays were carried out at room temperature in a single step 60 min incubation of immunoreagents with analyte. The assays were easy to perform and required a total time equal to the incubation period plus about ten minutes. The assay format is suitable for a wide range of biological antigens. New assays can be developed and optimized readily, often within one day. The apparatus employs, a light-addressable potentiometeric (LAP) sensor, is rugged and compact, and requires minimal logistic support. The work presented here demonstrates the utility of the LAP sensor for use in a field or remote hospital setting.

RÉSUMÉ

Un immunodosage de type sandwich rapide et non radioactif faisant appel à la capture par filtration d'immuncomplexes par l'intermédiaire du système biotine-streptavidine de concert avec un capteur au silicium a été mis au point pour la détection de virus et d'anticorps antivirayx dans le sérum. On a déterminé les limites inférieures de détection (LID) à l'aide du virus de la maladie de Newcastle (VMN) et des IgG anti-VMN de souris ajoutées dans du sérum de souris comme dans un modèle de type clinique. Les LID pour les sites de dosage ont été de 1 ng pour le VMN et de 4 ng pour les anti-VMN. Les dosages ont été réalisés à la température de la pièce en une seule étape au cours de laquelle les immunoréactifs et le substrat à analyser ont été incubés ensemble pendant 60 min. Les dosages ont été faciles à effectuer et le temps de dosage total a été égal à la période d'incubation plus environ 10 min. Le modèle de dosage convient à une grande variété d'antigènes biologiques. De nouveaux dosages peuvent être mis au point et optimisés rapidement, souvent en moins d'un jour. Le dispositif fait appel à un capteur LAP *(Light-addressable potentiometric sensor)*, il est robuste et compact et il ne nécessite presque pas de soutien logistique. Le présent document montre l'utilité du capteur LAP pour travailler sur le terrain ou dans un hôpital éloigné.

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Introduction

Clinical diagnosis of infectious disease is often made on the basis of monitoring the serum for the presence of infectious agent or for antibodies produced as a response to the infectious agent. Enzyme immunoassay is one of the principal methods employed in the diagnostic process. The strength of enzyme immunoassays, as an analytical or diagnostic technique, is derived from two biological phenomena; (i) the exceptional power of molecular recognition by antibodies and, (ii) the large catalytic or signal generating capacity of enzymes. Immunoassays have been used in a wide variety of formats for basic research and diagnostic purposes,[1] the most common format employing polystyrene microtitre plates, whereby antibody and antigen react at the polystyrene surface of the wells in the microplates. Although the association rate constants of antibody and antigen are large[2] (as high as 10^8 M^{-1} s^{-1}), in microplate assays the formation of an immune complex is generally rate limited by diffusion of macromolecular species (antibodies, proteins, polysaccharides, virus, bacteria etc.) to the reactive surface. Microtitre plate assays consist of a series of incubation, washing, and blocking steps that are time consuming and labour intensive. The advent of automated microplate washers has simplified the routine work of microplate assays somewhat but the time required is still in the order of three to four hours per plate.

A rapid immunoassay system employing a silicon-based biosensor, a light-addressable potentiometric (LAP) sensor, has been developed for the detection and quantitation of biological material in liquid samples.[3,4] The reagent antibodies and the antigen are incubated together in a single step. The resulting immune complexes are filtered through biotin-embedded nitrocellulose membrane and subsequently immobilized on the membrane by means of biotin-streptavidin interactions.[5,6] The presence of analyte on the membrane is detected by monitoring the enzyme activity associated with urease-conjugated antibodies in the immune complex. Monitoring is carried out by wetting the membrane with a solution of substrate, urea, and then placing the membrane in contact with the surface of the LAP sensor.

The LAP sensor has been used as a quantitative assay system for protein,[7,8] virus,[9] and

bacteria.[10] Assays have been shown to be sensitive, rapid and easy to peform, requiring only a few procedural steps. An automated LAP sensor has been developed for and employed in an environmental monitoring device, the Biochemical Detector.[11]

The present work describes the development of a clinical-style assay using the LAP sensor technology for detection. This clinical model system employs Newcastle disease virus (NDV) and anti-NDV immunoglobulin G in mouse serum. The availability of NDV and anti-NDV as well as the existence of a number of immunoassay studies[12,13] including a LAP sensor assay[9] and epitope mapping[14] of NDV, made this virus a logical choice as a model. As well, since NDV is nonpathogenic to humans and the vaccine strain employed herein is Agriculture Canada approved for dissemination into the environment, biological containment was not required. Although the ultimate aim of this research was to produce a clinical assay for medical diagnosis, initially mouse serum was employed in the model to avoid the health hazards associated with human serum. In order to establish quantitative levels of sensitivity and limits of detection, clinical-style samples were made by spiking known amounts of NDV or antibody to NDV into discrete volumes of serum. These were treated as clinical samples and were assayed accordingly.

Materials and Methods

Reagents

Bovine serum albumin (BSA), sodium dihydrogen phosphate, Tween 20, Triton X-100, urea, and goat anti-mouse IgG urease conjugate were obtained from Sigma Chemical Co. (St Louis MO) and used without any further purification. Prior to use in immunoassays the goat anti-mouse was diluted (1/1000) to a working concentration with assay buffer and filtered through 0.22 μm Millipore filters. Streptavidin was obtained from Scripps Laboratories (San Diego CA) and was reconstituted in distilled water to yield a concentration of 10 mg/mL. Hybridoma clones producing anti-NDV monoclonal antibodies were obtained under contract with the University of Alberta (Edmonton AB). Monoclonal antibodies against NDV were purified from ascites fluid using high-performance liquid chromatography as described previously.[12] Antibody labelling reagents, N-hydroxysuccinimide esters of carboxyfluorescein and dinitrophenylbiotin were obtained from Molecular Devices Corp. (Menlo Park CA) and were used according to procedures described in the product literature. Nitrocellulose membrane filters coated with biotinylated BSA, and anti-fluorescein urease conjugate were purchased from Molecular Devices. The latter was obtained in sealed vials as a lyophilized powder and was reconstituted by dissolving the contents in 30 mL of assay buffer. This solution could be stored at 4 °C for two weeks or longer. For use in immunoassays it was further diluted (1/10, v/v) with assay buffer. The viral antigen, NJ-La Sota strain of NDV, derived from seed stock of the American Type Culture Collection (Rockville MD) was supplied by the Molecular Biology Group (DRES). Protein concentrations were determined spectrophotometrically with a BCA protein assay kit (Pierce Chemical Co., Rockford IL). Mouse and guinea pig serum were obtained from Pel Freeze Biologicals (Rogers AR). Wash buffer consisted of 10 mM sodium phosphate pH 6.5, 150 mM sodium chloride, 0.2% Tween 20. Assay buffer was 10 mM phosphate sodium phosphate pH 7.0, 150 mM sodium chloride, 0.2% Tween 20, 0.25% Triton X-100, 0.1% BSA, 1% Bacto dehydrated skim milk (Difco Laboratories, Detroit MI).

Apparatus

The apparatus for the immunoassays was a commercially available LAP sensor, marketed under the name Threshold Unit™ and was purchased from the manufacturer, Molecular Devices Corp. The instrument was controlled by an IBM PS/2 model 30 microcomputer and custom designed software supplied by Molecular Devices. The assay system was capable of processing (filtration, potentiometric sensing) eight samples simultaneously.

Immunoassay Procedures

Figure 1 provides a schematic representation of the sandwich immunoassay employed for detection of NDV. Reagent solution consisting of 25 μL of biotin-labelled anti-NDV (2 μg/mL) and 25 μL of fluorescein-labelled anti-NDV (4 μg/mL) was added to a NDV-containing sample of volume 50 μL, mixed well and incubated at room temperature for 60 min. Five minutes prior to the end of the incubation period a 5 μL aliquot of streptavidin solution (5 μg/mL) was added to the reaction mixture and thoroughly mixed. Upon completion of the incubation, the reaction mixture was delivered to the well of the filter assembly of the Threshold Unit, filtered at 130 μL/min through biotinylated nitrocellulose membrane and rinsed with 500 μL of wash buffer (filtered at 750 μL/min). A volume of 1000 μL of dilute anti-fluorescence-urease conjugate was added to each well and filtered at 130 μL/min. The membranes were given a final rinse with 1000 μL wash buffer and filtered at 750 μL/min.

After the filtration capture procedures, the membrane stick which had immobilized urease-containing immune complexes was inserted into the reader compartment which housed the LAP sensor and substrate solution. A plunger pressed the membrane against the surface of the silicon sensor. The instrument was designed so that the spots on the membrane which contained the immobilized immune complex aligned with the pH-sensitive measurement sites on the surface of the LAP sensor. The data points were recorded and stored on the microcomputer using the manufacturer supplied software. The rate of change of pH with respect to time was monitored by the rate of change with respect to time of the surface potential as μVs^{-1}.

The immunoassay used for detection of mouse anti-NDV is depicted in Figure 2. The

capture agent was biotin-labelled NDV. An aliquot of 50 µL biotin-labelled NDV (1 µg/mL) was added to a sample of anti-NDV, 50 µL in volume, mixed well and incubated at room temperature for 60 min. Five minutes prior to the end of the incubation period a 5 µL aliquot of streptavidin solution (5 µg/mL) was added to the reaction mixture and thoroughly mixed. Upon completion of the incubation, the reaction mixture was delivered to the well of the filter assembly of the Threshold Unit, filtered at 130 µL/min through biotinylated nitrocellulose membrane and rinsed with 500 µL of wash buffer and filtered at 750 µL/min. A volume of 1000 µL of dilute anti-mouse IgG-urease conjugate (stock solution diluted 1/1000) was added to each well and filtered at 130 µL/min. Finally each well was rinsed with 1000 µL of wash buffer and filtered at 750 µL/min. After the filtration steps, the membrane was inserted into the reader compartment as described above and was monitored for urease activity.

Results

Immunoassay of Newcastle Disease Virus

Sandwich immunoassays of NDV samples reconstituted in buffer were carried out. The response of the LAP sensor was a monotonic increase in signal over a large range of analyte concentration, 0 - 250 ng per well (data not shown). The dose response to a set of standards, 0 - 20 ng per well, is shown in Figure 3. The set of standards on the calibration curve was run simultaneously on a single capture membrane stick. Each data point is the mean of three consecutive assays performed on the same day using the same reagents. The data, $\mu V.s^{-1}$ versus ng of antigen, was well represented by a linear plot. The error associated with the individual points was small: the coefficient of variation (cv), the ratio of the standard deviation (SD) to mean, was about 15%. The lower limit of detection was defined as the point on the calibration curve having an output signal equal to the background signal plus two standard deviations of the background. At the low concentration regime of the dose response curve the standard errors on the background were approximately equal to the standard errors of the samples. For the data given in Figure 3, the LOD was determined to be 1 ng and was comparable to previous LAP sensor assays for NDV.[9]

Immunoassays were carried out for NDV reconstituted in mouse serum. In general, the sensitivity of the assay (as indicated by the slope of the dose response curve, $\Delta\mu V.s^{-1}/\Delta ng$) was less for NDV reconstituted in serum than in buffer. Figure 4 provides an example of a serum-based assay. A monotonic dose response curve was indicated, however the scattering of the points about the calibration curve was higher than for NDV in buffer. Along with the decrease sensitivity of the serum-based assay was an increase in LOD, approximately 10 ng per well.

Biotin-labelled NDV reconstituted in both buffer and serum was assayed in a similar manner to the above, except that the capture antibody (i.e., biotin-labelled anti-NDV) was not required. In each preparation of biotin-labelled NDV, buffer and serum, LOD values were similar to native virus, approximately 1 and 10 ng per well, respectively (data not shown). Thus the antigenicity of NDV was not significantly affected by attachment of the biotin label.

Immunoassay of Mouse Anti-NDV IgG

Immunoassays of mouse anti-NDV IgG reconstituted in buffer were performed according to the reaction scheme given in Figure 2 which employed biotin-labelled NDV as the capture agent. The efficacy of the biotin-labelled NDV as a capture agent for anti-NDV was indicated above by the similarity in the detection limits of biotin-labelled and native NDV. A calibration curve of a preparation of monoclonal 25R5 analyte is shown in Figure 5 and LODs were determined to be about 0.12 ng per well. A similar assay of the monoclonal 55R3, had a LOD of 0.5 ng per well (data not shown). The differences in LOD for the two monoclonals may be a reflection of the avidity of the antibody toward the virus. For comparison, a sandwich LAP sensor immunoassay for mouse immunoglobulin G, employing biotin-labelled anti-mouse IgG for capture and urease-labelled anti-mouse IgG for detection, provided a LOD of 0.01 ng per well.[8]

Clinical-style immunoassays of mouse anti-NDV IgG, i.e., anti-NDV reconstituted in naive mouse serum, proved to be more difficult to carry out than the assay of anti-NDV reconstituted in buffer. The principal problem here, as in most clinical assays, was the presence of large amounts of mouse immunoglobulins from the serum which interacted with the detector antibody (anti-mouse IgG-urease conjugate) to a similar extent as did the analyte (anti-NDV).

Non-specific binding of the serum-derived mouse IgG to the nitrocellulose membrane rather than inhibition of the immunochemical interactions was the major source of the interference (data not shown).

It was found that the interference caused by the serum-based mouse immunoglobulins could be dimished by addition of guinea pig serum to the reaction mixture. The assay of mouse anti-NDV analyte in mouse serum utilizing guinea pig serum as a blocking agent is shown in Figure 6. The analyte was spiked into 10 µL mouse serum to give a set of standards ranging from 0 - 20 ng (as shown in Fig. 6). Prior to the addition of immunoreagent 20 µL of guinea pig serum was delivered each sample. The LOD of the assay was about 3-4 ng per well or 300-400 ng/mL.

Clinical diagnoses generally do not rely upon actual quantitation of infectious agents or antibodies to them. More often the relevant information is the degree of dilution that the agent or antibody can be detected above a control sample. Typically an immune response to an infectious agent will produce antibody to the infectious agent in excess of 100 µg/mL.[15,16] Serial dilutions of a clinical-style sample, i.e., mouse serum spiked with 100 µg/mL, were carried out and the results are shown in Table I. Using a cut-off of background plus two standard deviations analyte was detectible as low as 1/200 dilution, a concentration of 500 ng/mL (5 ng total mass per well). These results are in accordance with LODs determined from standard curves, as described above.

Discussion

In this report we have described sensitive nonradioactive immunoassays which have one-step liquid-phase incubation followed by a biotin-streptavidin mediated filtration-separation. The overall assay time was governed by the time allotted to incubation of the analyte and the immunoreagents. Maximal sensitivity was generally obtained after about one hour incubation, however shorter incubation times in the range of 1 - 10 min have been demonstrated to be effective, albeit with lesser sensitivity.[8,9] The LAP sensor assay system offered sensitivities comparable to convention microplate formats but the total time and number of steps required

were greatly reduced.

The use of biotin-streptavidin mediated immobilization allowed for generic capture. Antibody or virus, suitably labelled with biotin, is readily immobilized on the biotin coated nitrocellulose. Each functioned well as capture agent. In the present work, nitrocellulose membranes (0.44 μm pore size) were coated with biotinylated BSA which, in addtion to providing immobilization, also helped reduce nonspecific protein binding to the surface. For samples containing particulate or cellular material, similar biotin-BSA-coated nitrocellulose membranes with 5 μm pore size are available and have shown to be effective.[11] In general nonspecific binding in LAP sensor assays was not a problem, and neither was the case herein for detection of NDV (in buffer or serum) or anti-NDV (in buffer). However, the clinical-style assays of mouse anti-NDV spiked into mouse serum presented the difficulty of detecting a small amount of the analyte mouse IgG (50 ng or less) in a milieu of serum-based IgG of about 1 mg/mL. Since detection was a consequence of the interaction of anti-mouse IgG urease conjugate with mouse IgG, adhesion of the serum-based IgG to the membrane caused the background (negative control samples) to be significantly higher than the background of the samples reconstituted in buffer. However, the nonspecific adhesion of extraneous mouse IgG in the serum-based samples was alleviated by addition of guinea pig serum (two-to-three-fold excess over mouse serum) to the reaction mixture.

The purpose of the *in vitro* study presented here was to determine quantitative levels of sensitivity for virus and anti-virus IgG in serum samples. The mouse serum/NDV model was employed because of the availability of high quality (purified) virus and high titre monoclonal antibodies. It provided a readily definable system. The detectible limits obtained were low enough to be physiologically relevant[17] and thus, these results suggest that the LAP sensor assay is suitable for monitoring serum in clinical samples.

The LAP sensor immunoassay device possesses a number of advantages for use as a diagnostic tool in a field or remote hospital setting. The commercially available apparatus is compact in size (about 1 cu. ft or 0.03 m^3), rugged (possessing few moving parts) and controllable by a lap-top microcomputer. The work station component used for filtration is

reliable. Two units have been operated for approximately 1000 h each over three to four years without need of maintenance. The membrane-reader cartridges which contain the silicon chip-LAP sensor have an estimated lifetime of about 12 months but are inexpensive enough to be considered semi-disposable and small enough in size (4 x 8 x 10 cm) be stocked with other consumables.

The assays are easy to perform and rapid, requiring about 10 min of operator time plus the incubation period. In contrast to microplate immunoassays, no pre-assay preparation, such as coating the plates with capture or blocking agents, is required. Overall the assays are easy to support logistically. Excellent results on the LAP sensor have been obtained using lyophilized immunoreagents and buffer salts,[18] which are stable at ambient temperatures for long periods of time. The development of immunoassays for other antigens by the methods described herein can be achieved quickly. Starting with at least one high titre antibody, the preparation of biotin and fluorescein conjugates, generation of dose response curves, antibody optimizations and limits of detection can be carried out in about 8 - 10 h.

In this work we have demonstrated the utility of the LAP sensor for immunoassay monitoring of clinical-style samples, namely virus and antibody in serum. Limits of detection of NDV and anti-NDV in mouse serum were 1 and 4 ng, respectively. These results, combined with the relative ease of operation, minimal logistical requirements, and durability of the hardware suggest a potential application in a field or remote hospital setting. The LAP sensor technology is the detection system in both the Biochemical Detector (BCD) and a Biological Integrated Detection System (BIDS). As such, technical advances in sensor development made in the progress of BCD or BIDS would accrue to clinical applications. The same apparatus has also been used in a wide variety of other detection applications of potential medical interest. These include rapid immunoassay of environmental samples for detection of protein,[7,8] virus,[9] and vegative[10] and sporilated bacteria,[19] measurement of organophosphates and related anti-cholinesterase compounds[20],[21] and gene probes.[22]

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Table I. Dilution of mouse anti-NDV (IgG) spiked into mouse serum

Dilution*	Concentration** ($\mu g.mL^{-1}$)	Signal ($\mu V.s^{-1}$)	
		mouse anti-NDV	background#
1/50	2	44 (2)	33 (3)
1/100	1	39 (2)	33 (2)
1/200	0.5	34 (3)	29 (2)

*Mouse anti-NDV IgG was spiked into mouse serum to give an initial concentration of 100 μg/mL and was serially diluted with assay buffer.

**Volume of sample was 10 μL.

#The background samples consisted of naive mouse serum serially diluted in the same assay buffer.

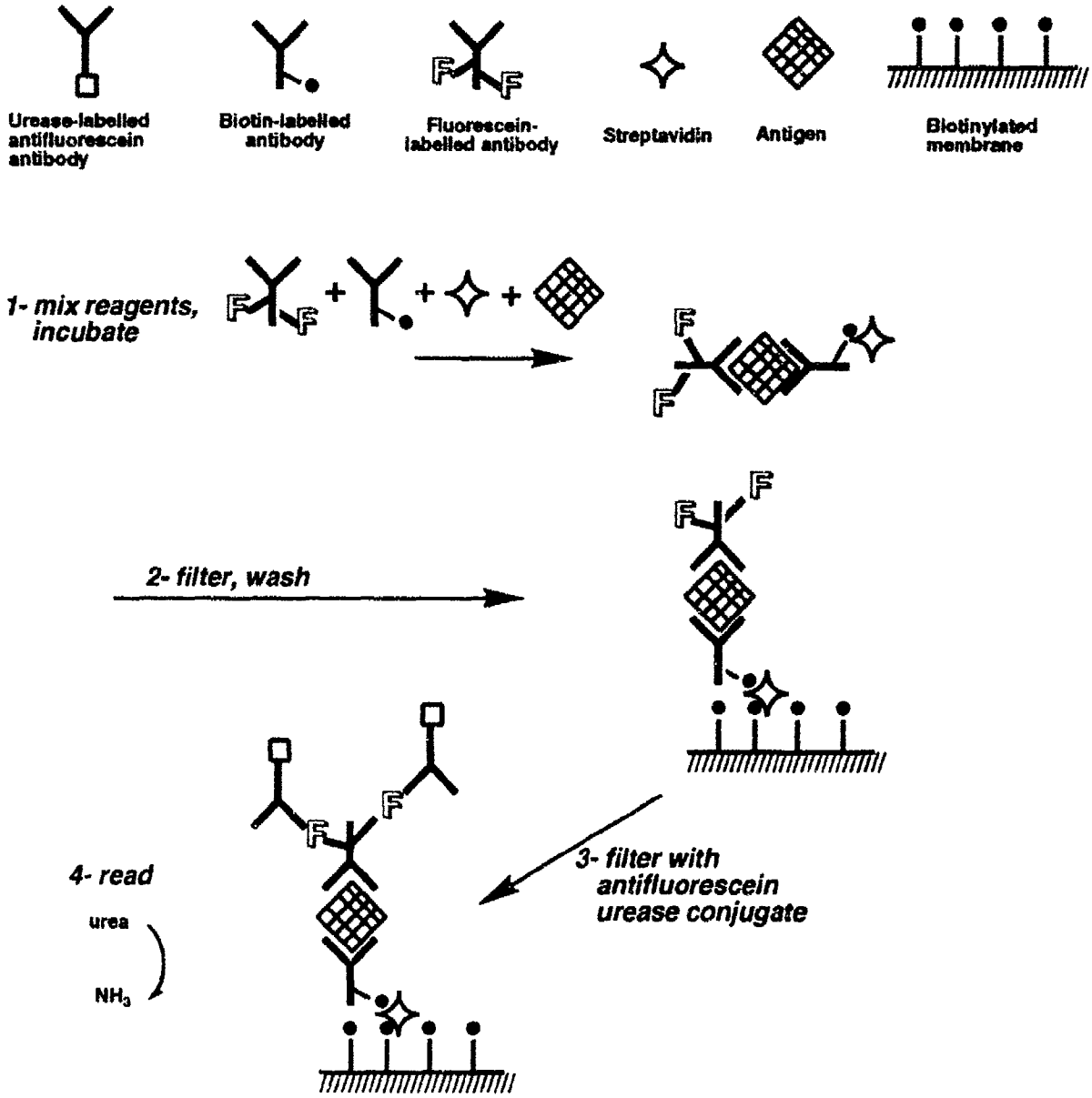

Figure 1. Scheme for immunoassays of NDV.

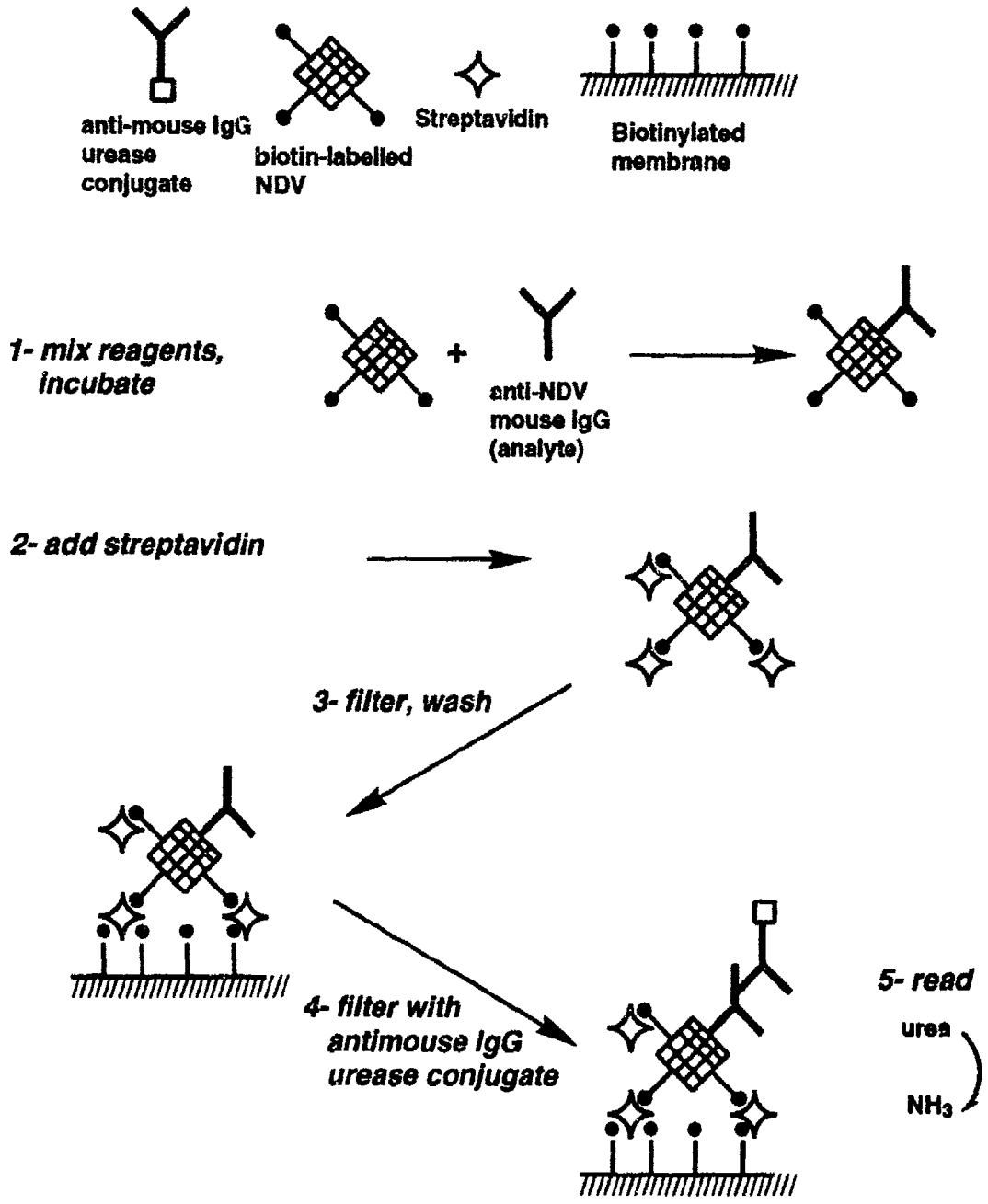

Figure 2. Scheme for immunoassays for antibody against NDV.

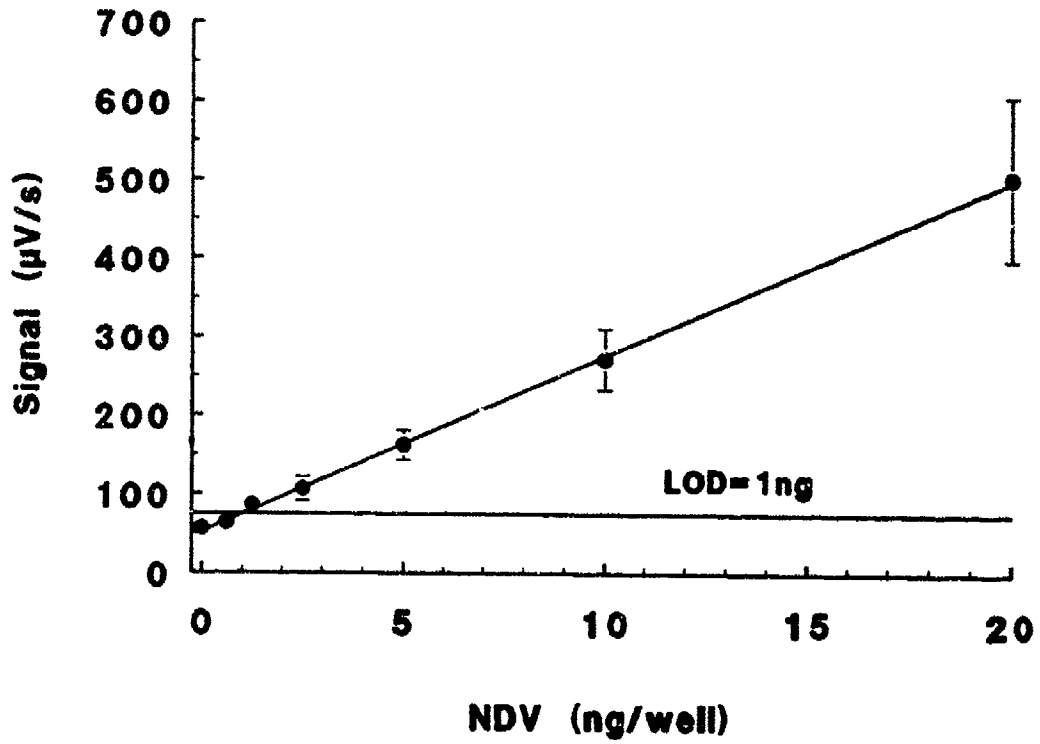

Figure 3. Standard curve of NDV in phosphate buffer employing immunoassay scheme depicted in Fig 1. Each point represents the mean of three independent determinations. Error bars, where not masked by the data point markers represent the +/- 1 SD. The LOD, taken to be the intersection of the calibration curve with the background (zero NVD) + 2 SD, was about 1 ng.

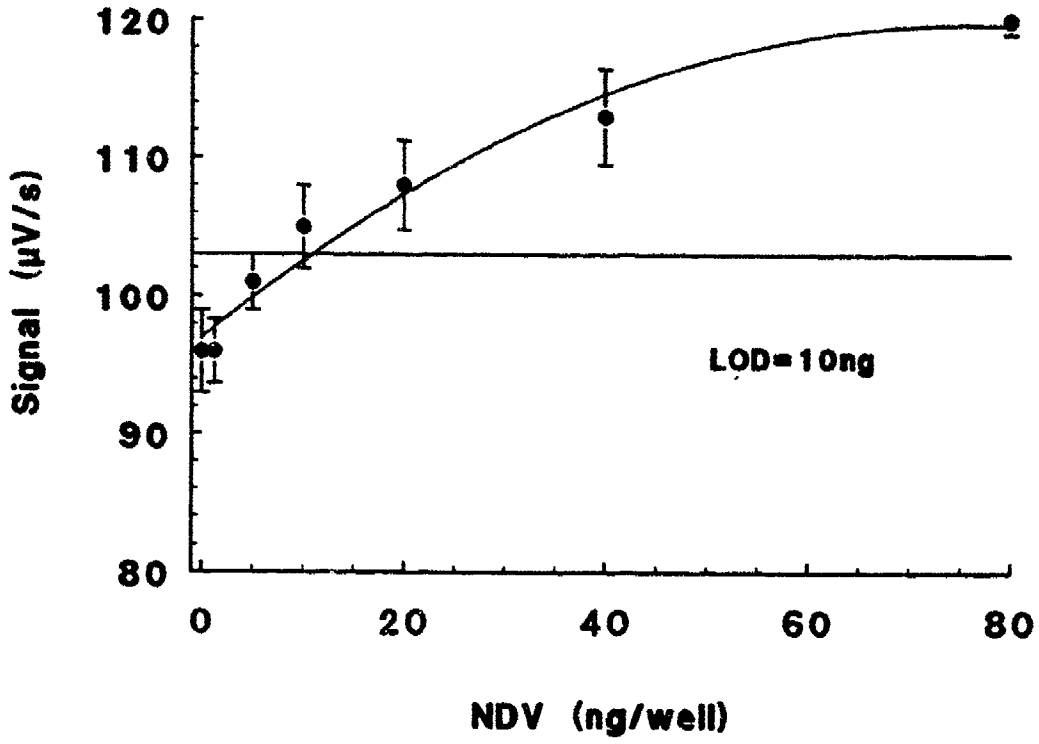

Figure 4. Standard curve of NDV in mouse serum employing immunoassay scheme depicted in Fig 1. Each point represents the mean of three independent measurements. Error bars represent +/- 1 SD. The LOD, taken to be the intersection of the calibration curve with the background (zero NVD) + 2 SD, was about 10 ng.

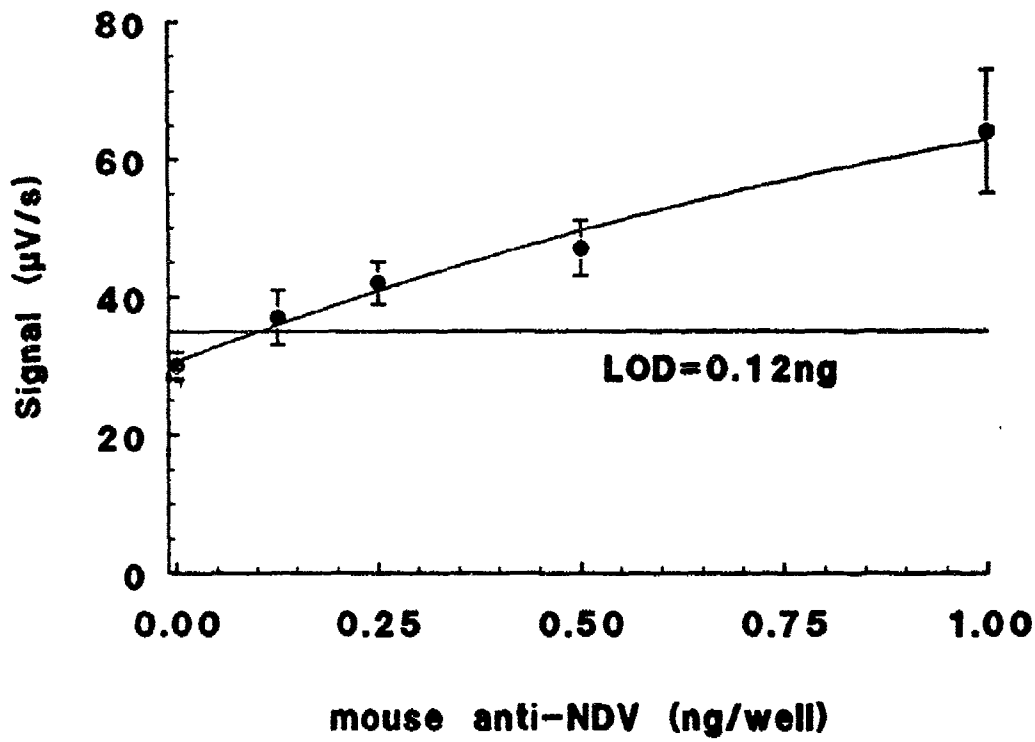

Figure 5. Standard curve of mouse anti-NDV (IgG) in phosphate buffer carried out by the immunoassay procedure shown in Fig 2. Each data point represents the mean of four independent measurements. Error bars represent +/- 1 SD. The LOD, taken to be the intersection of the calibration curve with the background (zero NVD) + 2 SD, was about 0.12 ng.

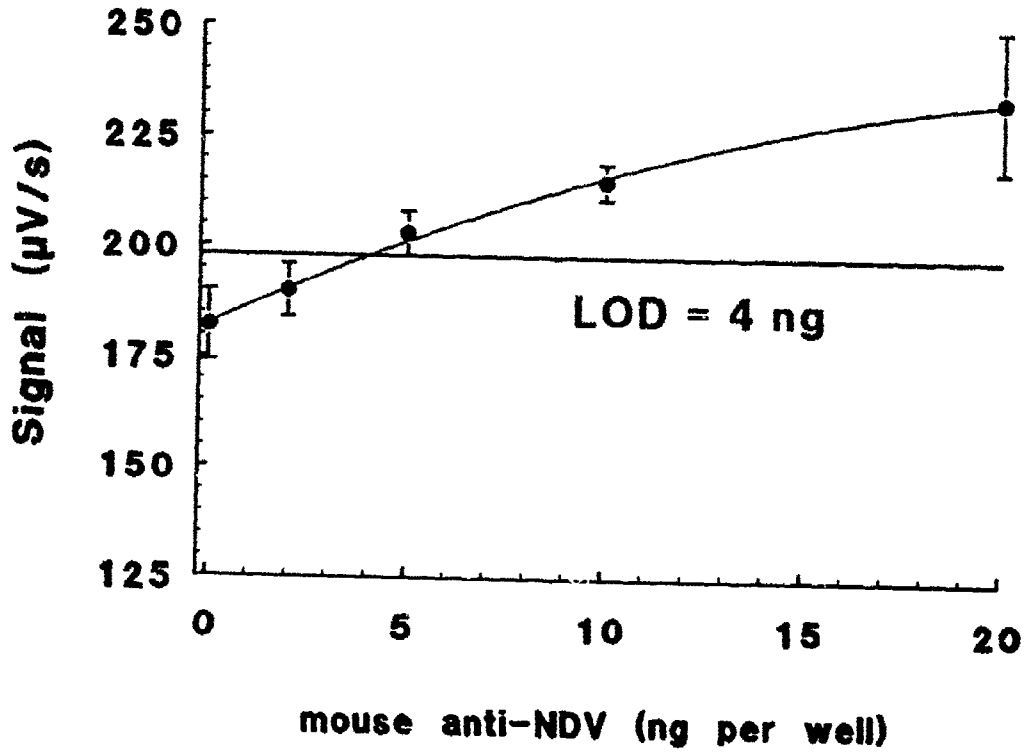

Figure 6. Standard curve of mouse anti-NDV (IgG) in mouse serum carried out by the immunoassay procedure shown in Fig 2. Each data point represents the mean of four independent measurements. Error bars represent +/- 1 SD. The LOD, taken to be the intersection of the calibration curve with the background (zero NVD) + 2 SD, was about 4 ng.

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A rapid non-radioactive sandwich immunoassay which utilizes biotin-streptavidin mediated filtration capture of immune complexes in conjunction with a silicon sensor was developed for the detection of virus and antibody to virus in serum. Using Newcastle Disease Virus (NDV) and mouse anti-NDV IgG spiked into mouse serum as a clinical-style model, the lower limits of detection (LOD) were determined. The LODs per test site were 2 ng for NDV and 4 ng for anti-NDV. The assays were carried out at room temperature in a single step 60 min incubation of immunoreagents with analyte. The assays were easy to perform and required a total time equal to the incubation period plus about ten minutes. The assay format is suitable for a wide range of biological antigens. New assays can be developed and optimized readily, often within one day. The apparatus employs a light-addressable potentiometric (LAP) sensor, is rugged and compact, and requires minimal logistic support. The work presented here demonstrates the utility of the LAP sensor for use in a field or remote hospital setting.

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Streptavidin
Biotin
Biosensor
Urease
Antibody
Newcastle Disease Virus
Detection
Serum